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(54) Title: PSYCHROPHILIC PROTEASE AND PSYCHROPHILIC BACTERIA

(57) Abstract

A novel psychrophilic protease and a microorganism having the psychrophilic protease producing ability are disclosed. The protease acts on and decomposes casein and dimethylcasein but not on ribonuclease, has an optimal temperature of about 40 °C. Under the condition of storage at pH 7 for 1 hour, it is inactivated scarcely at a temperature up to 30 °C, but at 40 °C it loses about 40 % of the activity. At 50 °C, the protease is rapidly inactivated so that the activity is completely abolished in about 15 minutes. *Flavobacterium balustinum* having the protease producing ability is also disclosed.

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PSYCHROPHILIC PROTEASE AND PSYCHROPHILIC BACTERIA

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a protease having a high activity at a low temperature range, its use and a psychrophilic bacterium producing the protease.

Background Art

Psychrophilic bacteria have been known for a long time, and their existence can be confirmed extensively in low temperature circumstances. Psychrophilic bacteria can be isolated from various low temperature circumstances such as soil, fishery products, milk products as well as artificial low temperature circumstances. Studies on psychrophilic bacteria have been conducted from the food microbiological requirement but principally confined to those with respect to the phylogeny of microorganisms.

Meanwhile, enzymes obtained from psychrophilic bacteria are expected to be the psychrophilic enzymes having an optimal temperature in a low temperature range. The psychrophilic enzyme which works efficiently at low temperatures is considered capable of being incorporated into for example a detergent which can be used even in water at a low temperature. It is also considered to be employed for the chemical reaction in the presence of an organic solvent which is volatile at the room temperature and for improving the quality of foods at a temperature that the foods will not be rotten. Furthermore, the study on the enzyme derived from the psychrophilic bacteria is fairly interesting to reveal the physiological functions and adaptation mechanism to a low temperature of the psychrophilic bacteria.

SUMMARY OF THE INVENTION

We have now found a novel bacterium strain which produces a novel psychrophilic protease.

Accordingly, an obj ct of the present invention is to provide a novel psychrophilic protease.

Another object of the present invention is to provide a novel microorganism which produces the psychrophilic protease.

Further object of the present invention is to provide a process for preparing the psychrophilic protease with the novel microorganism.

The psychrophilic protease according to the present invention has the following physicochemical properties.

- Specific activity and substrate specificity: the protease acts on casein and dimethylcasein to decompose them but does not act on ribonuclease.

- Optimal temperature: the protease has an optimal acting temperature at about 40℃.

- Temperature stability: under the condition of storage at pH 7 for 1 hour, it is scarcely inactivated at a temperature up to 30℃, but at 40℃ it loses about 40% of the activity, and at 50℃ it is rapidly inactivated so that the activity is completely abolished in about 15 minutes.

Furthermore, according to the preferred embodiment of the present invention, the present protease also has the following physicochemical properties:

- Optimal pH: the protease acts optimally at pH 7.5;

- Stable pH range: the protease is stable at a pH in the range of 6.0 - 10.0 under the condition of storage at 20℃ for 1 hour;

- Molecular weight: about 38 kDa (the SDS-PAGE and gel filtration methods);

- Isoelectric point: about 4.5.

Furthermore, the novel microorganism according to the present invention is Flavobacterium balustinum having the psychrophilic protease producing ability described above.

In addition, the process for preparing the psychrophilic protease according to the present invention comprises culturing Flavobacterium balustinum described above, and collecting the psychrophilic protease from the

culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the result of Example 2, or shows the relationship between temperature and the activity of proteases derived from the strain P104 and the protease, Subtilysin Carlsberg.

Figure 2 is a graph illustrating the result of Example 4 (2), or shows the influence of initial pH on the activity and growth of the extracellular protease of Flavobacterium balustinum P104.

Figure 3 is a graph illustrating the result of Example 4 (3), or shows the influence of culturing temperatures on the activity and growth of the extracellular protease of Flavobacterium balustinum P104. Figure 3 (A), (B) and (C) show the results at 10℃, 20℃, and 30℃, respectively.

Figure 4 is a graph illustrating the result of the elution by gel filtration in Example 5 (2) (b).

Figure 5 is a graph illustrating the result of the elution by chromatography in Example 5 (2) (c).

Figure 6 illustrates the result of SDS-PAGE for the measurement of molecular weight in Example 6.

Figure 7 is a calibration curve for the measurement of molecular weight in Example 6.

Figure 8 is a calibration curve of gel filtration for the measurement of molecular weight in Example 6.

Figure 9 illustrates the result of isoelectric focusing in Example 7.

Figure 10 is a calibration curve of isoelectric focusing in Example 7.

Figure 11 is a graph illustrating the result of Example 8, or shows the influence of pH on the enzyme reaction of the enzyme of the present invention.

Figure 12 is a graph illustrating the result of Example 9, or shows the stability of the enzyme of the present invention to pH.

Figure 13 is a graph illustrating the result of

Example 10, or shows the influence of temperature on the enzyme reaction of the enzyme of the present invention.

Figure 14 is a graph illustrating the result of Example 11, or shows the stability of the enzyme of the present invention to temperature.

Figure 15 is a graph illustrating the result of Example 13, or a graph illustrating the influence of the protein modifier SDS on the enzyme of the present invention.

Figure 16 is a graph illustrating the result of Example 13, or a graph illustrating the influence of the protein modifier urea on the enzyme of the present invention.

Figure 17 is Lineweaver-Burk plot of the enzyme of the present invention examined in Example 16. Figure 17 (A) and (B) show the change in 1/v value in the range of 0 to 2.0, and in the range of 0 to 0.2, respectively.

DETAILED DESCRIPTION OF THE INVENTION

Novel protease producing bacterium

The novel protease according to the present invention is produced by microorganisms which belongs to Flavobacterium genus and have the ability to produce a protease having the properties described above.

A specific example of the microorganisms having the ability to produce a protease according to the present invention preferably includes Flavobacterium balustinum P104. This strain is a microorganism isolated from the internal organs of salmon and has been deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as the deposit number of FERM BP-5006 on February 17, 1995.

The bacteriological properties of Flavobacterium balustinum P104 according to the present invention are listed in the following.

(1) Morphological property

The strain is in the form of short bacillus having a size of 0.4 - 0.5 × 1.7 - 1.9 μm.

(2) Nature on the culture medium

The strain grew on an agar medium and produced a yellow pigment.

(3) Optimal condition of growth

pH: The strain grew at a pH in the range of 5 - 9, and the optimal pH for growth was around neutrality.

Temperature: The strain grew at a temperature in the range of 10 - 30℃, and the optimal temperature for growth was around 20℃.

(4) Distinction between aerobic and unaerobic bacteria: aerobe.

(5) Gram stain: negative.

(6) Biochemical properties

<u>Flavobacterium</u> <u>balustinum</u> P104 had the main biochemical properties shown in Table 1 below.

Table 1

Test items	Results
Galactosidase	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Utilization of citric acid	-
Production of hydrogen sulfide	-
Urease	+
Tryptophan aminase	-
Production of indoles	+
Gelatinase	+
Glucose	-
D-mannitol	-
Inositol	-
D-sorbitol	-
Rhamnose	-
Sucrose	-

D-melibiose	-
D-amygdalin	-
L-arbinol	-
Oxidase	-

Although the strain seemed to be judged as Flavobacterium indolgenes from these properties, it was judged suitable to be classified into Flavobacterium balustinum by the comparison of the base sequence of DNA coding for 16S ribosomal RNA as is below described in Example 3 with the base sequence in a known microorganism.

For the culture of the strain, the culture medium may be either liquid or solid, but shaking culture or aeration culture with a liquid culture medium is generally used.

The culture medium for culturing the microorganism may be any one which is suitable for growth and can produce protease. Specifically, examples of the carbon source include glucose, trehalose, fructose, maltose, sucrose, starch, and malt oligo-saccharides. Examples of the nitrogen source include yeast extract, malt extract, beef extract, soybean powder, cotton seed powder, corn steep liquor, various amino acids and their salts, and nitrates. It is also possible to use synthetic media or natural media which contain properly inorganic salts such as magnesium, calcium, sodium potassium, iron and maganese phosphate, and the other nutrients according to necessities.

Culturing conditions such as the pH and temperature may be determined within the ranges of producing protease, liquid shaking culture or aeration agitation culture is preferably carried out at a pH around neutrality and at a temperature of about 20℃.

The protease of the present invention is produced in the cell wall of the bacterial cell, within the cell, and in the supernatant of the culture solution, and may be in either form f the bacterial cell, a crude enzyme

obtained from the bacterial cell or the supernatant of the culture solution, or of an extracted and purified enzyme. It is also possible to be in the form of protease immobilized on a substrate by the well-known method.

Collection of the Enzyme

In order to collect and purify the protease according to the present invention from the culture solution, the well-known methods can be used alone or in combination thereof.

The protease according to the present invention is mainly excreted extracellularly, namely into a culture solution, so that the bacterial cell can be easily removed for example by filtration or centrifugation to obtain a crude enzyme solution. The crude enzyme solution can be further purified by a known method. The method includes preferably the salting-out method with a salt such as ammonium sulfat; the precipitation method with an organic solvent such as methanol, ethanol or acetone; the adsorption method with raw starch; the ultrafiltration method; and a variety of chromatographical methods such as gel filtration chromatography or ion exchange chromatography. Specific examples of the preferred methods are described in Examples below.

Property of Protease

The property of the protease according to the present invention was examined, and the results are shown below.

(1) Activity and substrate specificity

The present enzyme decomposed well macromolecular proteins such as casein or dimethylcasein or denaturated proteins. It also decomposed gelatin which is the denaturated protein of collagen in a proportion of about 50% as compared with the case of casein. It acted however little on the other natural proteins, and it did not act at all particularly on ribonuclease.

Industrially used proteases such as subtilisin may have no substrate specificity and act on almost of

proteins. On the contrary, the enzyme of the present invention acts only on macromolecular proteins or on denaturated proteins into which the enzyme gets comparatively easily.

A protease which resembles the enzyme of the present invention and derived from psychrophilic bacterium, Pseudomonas fluorescens, has successfully decomposed macromolecular proteins such as casein or dimethylcasein or denaturated proteins. However, the protease, which is distinct from the enzyme of the present invention, also decomposes natural globular proteins such as hemoglobin and bovine serum albumin in a proportion of about 40% as compared with the case of dimethylcasein. Thus, the enzyme of the present invention is likely to have a substrate specificity higher than the well-known enzymes.

(2) Optimal temperature and stable temperature

The enzyme of the present invention acts at a temperature of about 40℃.

Under the condition of storage at pH 7 for 1 hour, it is scarcely inactivated up to 30℃, but at 40℃ it loses about 40% of the activity. At 50℃, it is rapidly inactivated so that the activity is completely abolished in about 15 minutes.

The enzyme of the present invention thus is the so-called psychrophilic enzyme which exhibits efficiently catalytic action at a low temperature.

(3) Optimal pH and stable pH range

The enzyme of the present invention has an optimal pH of 7.5.

Furthermore, it is stable at a pH in the range of 6.0 - 10.0 under the condition of retention at 20℃ for 1 hour.

The enzyme is thus a neutral protease, which will not work in an extremely acidic or alkaline range. Furthermore, it will be inactivat d during storage in an extremely acidic or alkaline range.

(4) Molecular weight

The enzyme of the present invention has a molecular weight of about 38 kDa as measured by SDS-PAGE and gel filtration methods.

(5) Isoelectric point

The enzyme of the present invention has an isoelectric point of about 4.5 as measured by isoelectric focusing.

(6) Inhibition of activity

The protease activity of the enzyme is not inhibited by phenylmethyl-sulfonyl fluoride or iodoacetamide, but inhibited noticeably by ethylenediaminetetraacetic acid, 2,2-bipyridyl, citric acid or oxalic acid. The protease activity is thus found to depend on a metal ion, so that it is suggested that the enzyme of the present invention is a metal protease. It is also considered from the inhibition of the protease activity by either of citric acid and oxalic acid that the activity depends on calcium.

In addition, the activity of the enzyme is inhibited noticeably by metal ions such as Ag^{+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Fe^{2+}, noticeably inhibited by Ag^{+} inter alia. However, it did not inhibited by either Mg^{2+} or Ca^{2+}.

(7) Enzyme reaction kinetics

The enzyme of the present invention shows the Michaelis-Menten type reaction rate to the concentration of a substrate such as casein. The Km value decreases and the Vmax value increases along with the increase of temperature. Moreover, the Kcat value of the enzyme exhibits a remarkably high value in the range from 10 to 40℃. An enzyme is generally tends to be inhibited at an excessive amount of substrates. However, the Kcat value increases when the system approaches the optimal working temperature in the case of the enzyme of the present invention. The enzyme is therefore advantageous in the point that appreciable inhibition will not be observed by decomposed products. It is also believed from the

Lineweaver-Burk plotting that inhibition due to temperature is of a mixed form, that is, the influence of temperature is of non-competitive inhibition or uncompetitive inhibition and the influence of decomposed products is of competitive inhibition.

Use of enzyme

The psychrophilic protease according to the present invention has an optimal temperature at a low temperature range. Thus, according to the psychrophilic protease of the present invention, it is possible to decompose a protein in a low temperature. For instance, a detergent which can be used even in water at a low temperature is prepared by incorporating the protease of the present invention into a detergent composition. This detergent composition can be prepared according to the conventional method except that the psychrophilic protease of the present invention is incorporated. Briefly, it can be prepared by combining the protease of the present invention with an ordinary detergent component such as a surface active agent for detergent, a bleach or a bilder.

Furthermore, the enzyme reaction of the psychrophilic protease according to the present invention can be carried out at a low temperature. Thus, even if the reaction system involves an organic solvent which is volatile at a room temperature, the reaction can be conducted at a low temperature where the organic solvent will not be volatilized. Moreover, when the quality of a food is intended to be improved by the protease according to the present invention, it is advantageous to employ the protease of the present invention because the reaction proceeds at a low temperature on which the food can be effectively prevented from decomposition.

Furthermore, since the protease according to the present invention is provided, advance in the study of the physiological mechanism of psychrophilic bacteria and their application m chanism at a low temp rature is expected.

The pres nt invention is described below in more

details with reference to specific examples, but the present invention is not intended to be limited thereto.

In this context, proteins were quantitatively determined by the protein staining method, Bio-Rad protein assay, and the protein fractions of the eluate in the chromatographical procedure were determined by the absorption in the ultraviolet range at 280 nm unless otherwise specified.

In addition, the activity of protease was measured as follows.

(a) Decomposition activity of protein with azocasein

A 0.05 ml portion of a sample enzyme solution was added to 0.3 ml of a 0.067M phosphate buffer containing 1% (W/V) azocasein (pH 7.0), and the mixture was kept at 30℃ for 30 minutes. The reaction was then terminated with a 6% trichloroacetic acid solution. After 15 minutes, the reaction mixture was centrifuged at 14,000 rpm at room temperature for 5 minutes. The absorbancy of the supernatant at 340 nm was measured. The enzyme activity was defined on the basis of ACU (azocasein digestion unit) which means the increase of absorbancy of 0.001 per minute at 340 nm.

(b) Decomposition activity of protein by the modified Anson method

A 0.05 ml portion of a sample enzyme solution was added to 0.3 ml of a 0.067M phosphate buffer containing 1% (W/V) protein (pH 7.0), and the mixture was kept at 30℃ for 30 minutes. The reaction was then terminated with a 7.5% trichloroacetic acid solution. After 30 minutes the reaction mixture was centrifuged at 14,000 rpm at room temperature for 5 minutes. The absorbancy of the supernatant at 280 nm was measured. The enzyme activity was defined on the basis of AU (modified Anson unit) which means the production of tyrosine in an amount of 1 μmole per minute.

Example 1

(1) Screening of novel bacterial strains

Isolation of a novel bacterial strain was conducted on an agar plate medium. An isolated sample of internal organs of salmons was suspended in aqueous physiological saline, and the supernatant was used as a stock solution. A 10^2 dilution was prepared from the stock solution. A 0.2 ml portion of each of the stock solution and the 10^2 dilution was sprayed on an agar plate medium for screening (3 g/liter of polypeptone, 10 g/liter of yeast extract, 10 g/liter of sodium casein, 0.2 g/liter of $MgSO_4 \cdot 7H_2O$, 2.0 g/liter of agar, on a 9 cm Petri dish), and cultured at 10℃ for 3 days. Colonies grown well among the colonies which had been grown on the agar plate were selected and subcultured as well as inoculated on an agar plate for stock.

The activity of an exoenzyme was assayed on an agar plate medium. The bacterial strain isolated was inoculated on an agar plate medium for screening as described above by streaking and cultured at 10℃ for 3 days. A 10% trichloroacetic acid solution was then sprayed on the agar plate medium on which the bacteria were grown to assay the protease producing bacterium by the presence of clear plaques.

(2) Culturing of the isolated bacterial strain and production of an enzyme

The isolated bacterium from the stock medium was inoculated on 25 ml of a pre-culture medium (10 g/liter of polypeptone, 10 g/liter of endoextract, 0.2 g/liter of $MgSO_4 \cdot 7H_2O$, pH 7.0, in a 100 ml Erlenmeyer flask) and rotary-shake cultured at 10℃ at 150 rpm for 48 hours in TAITECNR-80 for stabilizing the growth activity of the bacterium. In the regular culture, 0.25 ml of the pre-culture solution was inoculated on 25 ml of a medium for producing enzymes (5 g/liter of polypeptone, 2.5 g/liter of yeast extract, 5 g/liter of sodium casein, 0.2 g/liter

of $MgSO_4 \cdot 7H_2O$, pH 7.0, on a 100 ml Erlenmeyer flask) and rotary-shake cultured at 10℃ at 150 rpm in a rotary shaker for 72 hours. The culture medium had been previously steam-sterilized at 1.2 kgf/cm^2 gauge (121℃) for 15 minutes.

The bacterial strain isolated was stored in an agar plate medium for storage at 10℃, and subcultured after 2 weeks - 1 month.

(3) Measurement of protease activity

The culture solution obtained in the preceding step (2) was clarified by centrifugation (17,000 × g, 4℃, 15 minutes). The supernatant was used as a crude enzyme solution. The protease activity was measured by the decomposition of azocasein. A 0.05 ml portion of the crude enzyme solution was added to 0.3 ml of a 0.067M phosphate solution containing 1% (W/V) azocasein (pH 7.0), and the mixture was kept at 20℃ for 30 minutes. The reaction was then terminated with a 6% trichloroacetic acid solution, and after 15 minutes the reaction mixture was centrifuged at 14,000 rpm at room temperature for 5 minutes. The absorbancy of the supernatant at 340 nm was measured with a spectrophotometer (Beckman DU640). The enzyme activity was defined on the basis of ACU (azocasein digestion unit) which means the increase of absorbancy of 0.001 per minute at 340 nm.

As the result of procedures in (1) - (3), the bacterial strain P104 having a protease activity was isolated. The bacterial strain had a protease activity shown in the following table.

In the table, the growth rate of the strain was obtained by comparing with the growth of the divided strain Cytophaga xantha IFO 14972.

Table 2

Bacterial strain having protease activity

Strain	Growth rate	Protease activity ($\times 10^3$ ACU/ml)
P104	+++	0.982

Example 2

Protease activity of P104

Influence of temperature on enzyme activity was examined with a culture solution of the protease producing strain P104 in the temperature range of 0 - 60℃. Temperature dependency of enzyme activity was also examined in the same way with Subtilisin Carlsberg (Sigma) which is a commercially available enzyme protease derived from Bacillus licheniformis.

The results are shown in Figure 1, in which the specific enzyme activities for the strain P104 is represented by □ and the specific enzyme activities for Subtilisin Carlsberg is represented by △.

The optimal temperature was 40℃ for the exoprotease of the strain P104 and 60℃ or more for Subtilisin Carlsberg, respectively. The exoprotease of the strain P104 retained the protease activity at 40% or more of the activity at the optimal temperature at a temperature of about 20℃. Subtilisin Carlsberg retained only about 10% of the protease activity at an optimal temperature.

Further, activation energy of the enzyme reaction of these exoproteases was calculated in the range from 10 to 40℃. The results are shown in the following table.

Table 3

Bacterial Cell	Activation Energy (kJ/mole)
P104	39.8
Bacillus subtillis	58.3

Example 3

Identification of the strain P104 by the base sequence of DNA coding for 16S ribosomal RNA

The culture solution obtained in Example 2 was sampled in a 1.5 ml microtube, and the bacteria was collected by centrifugation. Genomic DNA was extracted to amplify the base sequence of DNA coding for 16S ribosomal RNA by PCR (polymerase chain reaction). The base sequence was then determined using the Sanger method, compared with the data base of GenBank for identification. The primers used are listed below, and 1F-Link and 5R-Link were used as PCR.

Primers:

1F-Link: 5'-TGTAAAACGACGGCCAGTAGTTTGATCATGGCTCAG-3';

3R-Link: 5'-CAGGAAACAGCTATGACCCGTCAATTCATTTGAGTT-3';

3F-Link: 5'-TGTTAAAACGACGGCCAGTGTAGCGGTGAAATGCGTA-3';

5R-Link: 5'-TGTAAAACGACGGCCAGTAAGTCCCGCAACGAGCGCAA-3'.

Results of comparison with the data base of GenBank are shown in the following tables. In the tables, Query represents 16S ribosomal RNA gene derived from the strain P104, and Subject represents Flavobacterium balustinum 16S ribosomal RNA (FVBRR16SH). As a result, the bacterial strain was identified as Flavobacterium balustinum. The strain P104 is thus referred as Flavobacterium balustinum P104.

(a) Used primer 1F-Link

Identities = 185/204 (90%), Positives = 185/204 (90%)

```
Query:1     G A T G A A C G C T A G C G G G A G G C
            : : :   : : : : : : : : : : : : : : : :
Sbjct:31    G A T N A A C G C T A G C G G G A G G C
Query:21    C T A A C A C A T G C A A G C C G A G C
            : : : : : : : : : : : : : : : : : : : :
Sbjct:51    C T A A C A C A T G C A A G C C G A G C
Query:41    G G T A T T T G T C T T T C G G G A C A
            : : : : :       :     : : : : :   :
Sbjct:71    G G T A T A G A T T C T T C G G A A T C
Query:61    G A G A G A G C G G A G T A C G G G T G
              : : : : : : : : :   : : : : : : : : :
Sbjct:91    T A G A G A G C G G C G T A C G G G T G
Query:81    C G G A A C A C G T G T G C A A C C T A
            : : : : : : : : : : : : : : : : : : : :
Sbjct:111   C G G A A C A C G T G T G C A A C C T A
Query:101   C C T T T A T C A G G G G G A T A G C C
            : :   : : : : : : : : : : : : : : : : :
Sbjct:131   C C N T T A T C A G G G G G A T A G C C
Query:121   T T T C G A A A G G A A G A T T A A T A
            : : : : : : : : : : : : : : : : : : : :
Sbjct:151   T T T C G A A A G G A A G A T T A A T A
Query:141   C C C C A T A A T A T A T T G A T T G G
            : : :   : : : : : : : : : : : :   : : :
Sbjct:171   C C C N A T A A T A T A T T G A C T G G
Query:161   C A T C A G T T A C T A T T G A C A A C
            : : : : : : :       : : : : : :   : : :
Sbjct:191   C A T C A G T C G A T A T T G A A A A C
Query:181   T C C G G T G G A T A G A G A T G G T C
            : : : : : : : : : : : : : : : : : :   :
Sbjct:211   T C C G G T G G A T A G A G A T G G G C
Query:201   A C G C
            : : : :
Sbjct:231   A C G C
```

(b) Used primer 3F-Link

Identities = 208/330 (63%), Positives = 208/330 (63%)

```
Query:1     G A T A T T A C T T A G A A C A C C A A
            : : : : : : : : : : : : : : : : : : : :
Sbjct:695   G A T A T T A C T T A G A A C A C C A A
Query:21    T T G C G A G G G A N G T T C A C T A T
            : : : : : :   : :     :   : : : : : : :
Sbjct:715   T T G C G A A G G C A G G T C A C T A T
Query:41    G T N T N A C C T G A T G C N G A T G N
            : :   :   :   : : : :   : :   : : : :
Sbjct:735   G T T T T A A C T G A C G C T G A T G G
Query:61    C C G A A A G T G N G N T G A G T G A A
              : : : : : :   :   :     : : :   : : :
Sbjct:755   A C G A A A G C G T G G G G A G C G A A
Query:81    C A G G A T T A G T T N C C A T G G T C
            : : : : : : : : :   :   : :   : : : :
Sbjct:775   C A G G A T T A G A T A C C C T G G T A
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(c) Used primer 3R-Link

Identities = 128/162 (79%), Positives = 128/162 (79%)

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Example 4

Culture of Flavobacterium balustinum P104

(1) Measurement of the concentration of bacterial cell

The culture solution obtained in Example 2 was diluted with physiological aqueous saline to ensure that 0 - 5 cells were contained in a bacterial counter cell. The cells were countered with an optical microscope. The turbidities of the culture dilutions were measured spectroscopically at 660 nm to obtain the correlation between the cells and turbidity. The relationship between the turbidity and the bacterial cell concentration of Flavobacterium balustinum P104 were represented by the following equation:

$$(\text{Cell/ml}) = 1.13 \times 10^9 \times \text{Abs. } 660 \text{ nm}$$

wherein

1.13×10^9: a factor obtained from the calibration curve.

(2) Influence of pH

The bacterial strain was cultured at various initial pHs of the protease producing culture medium in the range from 5 to 9 in order to examine the influence of the initial pH on exoprotease activity and growth of it.

The results are shown in Figure 2.

In an alkaline pH, the proliferation was significantly lowered and the protease activity was also lowered. However, insignificant difference was observed in either the proliferation or the protease activity in an acidic pH range. The bacterial strain proliferated best and the protease activity was maintained at the highest level in a neutral pH range.

(3) Influence of culturing temperature

Flavobacterium balustinum P104 was cultured at a various temperature in the range from 10 to 30℃ to examine the fluctuation of the exoprotease activity and the proliferation with the passage of the cultur .

The results ar shown in Figur 3.

While the bacterial strain was grown well at either temperature of 10℃ or 20℃, the proliferation rate at 30℃ was about half of that in a stationary state at 10℃ or 20℃. The strain exhibited the highest proliferation rate at 20℃, so that the optimal temperature for culture is believed to be about 20℃.

Example 5

Purification of protease derived from Flav. balustinum P104

(1) Culture of bacterial strain

The isolated bacterial strain obtained from the following stock culture medium was inoculated into 25 ml of the following pre-culture medium (in a 100 ml Erlenmeyer flask) and rotary shake-cultured at 150 rpm in TAITEC NR-80 at 10℃ for 48 hours. Regular culture was carried out by inoculating 0.25 ml of the pre-culture solution in 25 ml of the following regular culture medium in a 10 ml Erlenmeyer flask and rotary shake-culturing the solution at 150 rpm at 10℃ for 72 hours.

Stock medium:

Polypeptone	3 g/l,
Enzyme extract	2.5 g/l,
Sodium casein	20 g/l,
$MgSO_4 \cdot 7H_2O$	0.2 g/l,
Agar	20 g/l,
pH 7.0	

Pre-culture medium:

Polypeptone	3 g/l,
Enzyme extract	2.5 g/l,
Sodium casein	1 g/l,
$MgSO_4 \cdot 7H_2O$	0.2 g/l,
pH 7.0	

Regular culture medium:

Polypeptone	3 g/l,
Enzym extract	2.5 g/l,

Sodium casein	5 g/l,
$KH_2PO_4 \cdot 7H_2O$	3 g/l,
$MgSO_4 \cdot 7H_2O$	0.2 g/l,
pH 7.0	

Materials such as the culture media were sterilized with a high pressure steam in an autoclave at 1.2 kgf/cm^2 gauge (120℃) for 15 minutes.

Flav. balustinum P104 was stocked in an agar plate for stock at 10℃. It was subcultured for a period from 2 weeks to 1 month.

(2) Purification of protease

(a) Salting out with ammonium sulfate

The culture solution obtained in the preceding step (1) was clarified by centrifugation (17,000 × g at 4℃ for 15 minutes). The supernatant was used as a crude enzyme solution. Ammonium sulfate was added to the crude enzyme solution to ensure that the solution contained ammonium sulfate at 50% of the saturated concentration. After slow stirring for 1 hour, the solution was sedimented by centrifugation (17,000 × g at 4℃ for 15 minutes) to give a 0 - 50% saturation fraction. The added amount of ammonium sulfate in the saturated concentration at 25℃ was used as the amount ammonium sulfate added.

(b) Gel filtration

Gel filtration was next carried out on a HiLoad 16/60 Superdex 200 prep grade column. All of the operations in column chromatography were carried out at 4℃ with HiLoad System 50 (Farmacia Biotec Co.) as a chromatography system.

The HiLoad 16/60 Superdex 200 prep grade column was equilibrated by flowing a 20 mM Bis-Tris buffer (pH 6.0) at a linear rate of about 60 cm/hour in a proportion of at least 3 (400 ml) to the gel volume. A 5 ml portion of the sample enzyme solution which had been subjected to salting out with ammonium sulfate was load d on the column with a Superloop. The column was eluted with 20 mM Bis-Tris buffer

(pH 6.0) as an eluent at a linear rate of about 60 cm/hour to collect 5 ml fractions.

The elution curve is shown in Figure 4.

As the exclusion limit, a transparent yellow protein having a high molecular weight which had been contained in the culture solution was eluted. As the subsequent fraction having a ultraviolet absorption at 280 nm, a colorless transparent protein was eluted. The fragment attached to the protease seemed to removed because of the existence of protease activity in this fraction.

(c) Column chromatography

Ion exchange chromatography was next carried out with a Q Sepharose HP column. A column of ϕ 0.7 × 12.5 cm made up of 5 HiTrapQ (1 ml) columns connected in series was used as the chromatography column. The column was equilibrated by flowing a 20 mM Bis-Tris buffer (pH 6.0) at a linear rate of about 35 cm/hour in a proportion of at least 5 (25 ml) to the gel volume.

The sample enzyme solution of the fraction eluted by the gel filtration and having an protease activity was loaded on the column with a Superloop at a linear rate of about 17.5 cm/hour. The column was eluted with 80 ml of 20 mM Bis-Tris buffer having 1M NaCl added thereto by the linear ion strength increasing gradient (0 - 150 mM) at a linear rate of about 35 cm/hour to collect 2 ml fractions.

The elution curve is shown in Figure 5.

In addition, the purification procedure described above is summarized in the following table.

Table 4
Summary of the purification of protease

	Amount (ml)	Protein (mg)	Enzyme activity ($\times 10^3$ ACU)	Specific activity ($\times 10^3$ ACU $\cdot$ mg^{-1})	Recovery rate (%)	Purification degree ($\times$)
Cultured material	270	13.0	365	28.1	100	1
Salting out with ammonium sulfate	5	3.38	273	80.8	74.8	2.9
Gel filtration	25	0.752	77.7	103	21.3	3.7
Ion exchange chromatography	18	0.154	29.3	190	8.03	6.8

Example 6

Determination of the purity and molecular weight of protease

(1) Electrophoresis

(a) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A 10% polyacrylamide gel having a thickness of 1 mm was used. Electrophoresis was carried out by applying 20 mA of an electric current to the gel until Bromophenol Blue (BPB) reached the lowermost terminal. The gel plate was stained with an aqueous mixture of 30% methanol and 10% acetic acid having 0.02% Coomassie Brilliant Blue R250 dissolved therein for 1 hour and then decolored with a decolorant (30% methanol and 10% acetic acid) overnight.

The molecular weight of the protease was determined by SDS-PAGE with phosphorylase, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin as the markers.

The results and calibration curve of SDS-PAGE are shown in Figures 6 and 7. The enzyme exhibited a single band and thus is considered to be a single protein but not of a sub-unit structure.

(b) Gel filtration

The molecular weight was determined by the gel filtration method with Hiprep 16/60 Sephacryl S-100 HR. The

column was equilibrated by flowing a 50 mM phosphate buffer having 0.15 M NaCl added thereto (pH 7.0) at a linear rate of about 30 cm/hour in a proportion of at least 3 (400 ml) to the gel volume. A 1 ml portion of the sample enzyme solution was loaded on the column, and eluted with the same buffer as above at a linear rate of about 30 cm/hour to collect 2 ml fractions. The excluded volume was determined for Blue Dextran 2000 with albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa) as the standard proteins.

The results are shown in Figure 8. In addition, the ultra-violet absorption at 280 nm of the protein and the enzyme activity were accorded with each other. The ultra-violet absorption curve of the protein also exhibited a maximum absorption at 278 nm with no visible absorption. The protein was considered from these observations to have a purity satisfactory for examining the properties of it.

Example 7

Isoelectric focusing

Isoelectric focusing was carried out with a Phast system (Farmacia-Biotec Co.). IEF3-9 gel was employed, and the sample was loaded at the point on the gel at a distance of 3 cm from the anode. Electrophoresis was carried out under the condition of 2,000 V, 2.5 mA at 15℃ and 410 Vh. The gel plate was stained by fixing with a 20% TCA solution at 20℃ for 5 minutes, and washed with a rinsing and decoloring solution (30% methanol and 10% acetic acid) for 2 minutes. The plate was finally rinsed and decolored with a solution having 0.02% Coomassie Brilliant Blue R250 dissolved therein at 50℃ for 10 minutes. A Broad pI Calibration kit (Farmacia-Biotec Co.) was used as a pI marker.

The results and calibration curve of the isoelectric focusing are shown in Figures 9 and 10.

Th enzyme was stained substantially as a single band having an isoelectric point at pH 4.5.

Example 8

Influence of pH on enzyme reaction

Azocasein was decomposed with the enzyme at various pHs.

Buffer solutions in the reaction mixture had a concentration of 67 mM and comprised an acetate buffer (pH 4 - 5.5), KH_2PO_4-Na_2HPO_4 (pH 6.0 - 8.0), $Na_2B_4O_7$-HCl (pH 8.0 - 9.0), $Na_2B_4O_7$-NaOH (pH 9.5 - 10.0), and Na_2HPO_4-NaOH (pH 10.5 - 12.0), respectively.

The results are shown in Figure 11.

The relative activity of the enzyme at pH 7.5 as the optimal pH was maintained at a level of about 80% in the pH range from 6.0 to 10.0. The enzyme was thus found to act over a considerably wide range centering around neutral pH. However, the enzyme did not work quite satisfactory in the ranges of pH 5.5 or less or 10.5 or more, and it was inactivated at pH 12 to lost the protease activity.

Example 9

pH stability of protease

The enzyme was examined in buffer having various pHs with an Econo-Pac (Biorad Co.). Buffer solutions used had a concentration of 67 mM and comprised an acetate buffer (pH 4 - 5), KH_2PO_4-Na_2HPO_4 (pH 6 - 8), glycine-NaOH (pH 9 - 10), and Na_2HPO_4-NaOH (pH 11 - 12), respectively. After exchanging the buffer, the enzyme was incubated at 20℃ for 1 hour to evaluate the survived protease activity.

The results are shown in Figure 12.

It was found that the enzyme was stable over the range of pH from 6.0 to 10.0 under the condition at 20℃ for 1 hour, but inactivated at pH 4.0 and 12.0 under the same condition as above.

Example 10

Influence of temperature on enzyme reaction

Azocasein was decomposed with th enzyme at various pHs at a reaction temperature in th range from 0 to 70℃.

Similar reaction was conducted for commercially available enzymes such as Subtilisin Carlsberg, V8 protease which was the protease derived from Staphylococcus aurcub V8, Sabinase (Novonordisc) and Alkalase (Novonordisc).

The results represented by Kcat on the assumption that the substrate is present in an excess amount and thus the enzyme is all present in the form of an enzyme-substrate complex is shown in Figure 13, in which □ represents the enzyme, ◇ represents V8 protease, ○ represents Subtilisin Carlsberg, △ represents Sabinase, and ▽ represents Alkalase.

It was found that the enzyme had an optimal temperature at 40℃ and was rapidly inactivated at an enzyme reaction temperature over the optimal temperature. It was also found that all of the commercially available proteases had an optimal temperature of 50℃ or more and that the Kcat value of the present enzyme was higher than any of those of the comparative proteases in the range from 10 to 40℃.

Example 11

Temperature stability of protease

The present enzyme was maintained at a temperature in the range from 20 to 50℃. The variation of the activity with the passage of time is shown in Figure 14, in which □ represents the variation at 20℃, ◇ at 30℃, ○ at 40℃, and △ at 50℃.

The enzyme was scarcely inactivated at 20℃ or 30℃, but it is gradually inactivated at 40℃ to about 60% of the protease activity at 20℃ or 30℃ after 1 hour and rapidly inactivated at 50℃ to completeness in about 15 minutes.

The present enzyme is considered to be unstable to heat, since the above temperatures are lower than the optimal temperature of the comparative proteases employed in the preceding experiment.

Example 12

Influence of inhibitors

Phenylmethylsulfonyl fluoride (PMSF) acting on serine protease, iodoacetamide (IAA) acting on cysteine protease, ethylenediaminetetraacetic acid (EDTA) acting on metal protease, o-phenanthroline, 2,2'-bipyridyl, and a citrate and an oxalate specifically acting on calcium were employed as the inhibitors. After various concentrations of the inhibitors were added to the enzyme reaction system, it was maintained at 20℃ for 1 hour to examine the survived protease activity.

The results are shown in the following table.

Table 5

Influence by inhibitors

Inhibitor	Concentration	Survived activity (%)
None	10 mM	100
PMSF	10 mM	99
Iodoacetamide	10 mM	94
EDTA	10 mM	8
o-Phenanthroline	10 mM	91
2,2'-Bipyridyl	10 mM	39
Citrate	10 mM	41
	100 mM	0
Oxalate	10 mM	45
	100 mM	0

The protease activity of the enzyme was not inhibited by PMSF or IAA, but noticeably inhibited by EDTA, 2,2'-bipyridyl, a citrate or an oxalate. It was found from these observations that the protease activity is metal ion dependent, and thus the present enzyme is a metal protease. It is also considered from the inhibition by a citrate or an oxalate that the protease activity depends on calcium.

Example 13:

Influence by protein denaturing agents

SDS and urea were used as the protein denaturing agents. After various concentrations of the protein denaturing agents were added, the enzyme reaction system was maintained at 20℃ for 1 hour to examine the remaining protease activity.

The results for SDS and urea are shown Figures 15 and 16, respectively.

The protease was inhibited by SDS even in quite a low concentration and completely inactivated to completeness with 0.25% of SDS. The protease was not inhibited by urea in a concentration up to 2 M, but it was inhibited to about 40% by 3 M urea and completely inactivated by 4 M urea.

Example 14

Influence by metal ions

$AgNO_3$, $CuSO_4$, $ZnSO_4$, $CoSO_4$, $FeSO_4$, $MnSO_4$, $CaCl_2$ and $MgSO_4$ were employed as the metal sources. After the metal salt was added to ensure that the final concentration was 1 mM, the enzyme reaction system was maintained at 20℃ for 1 hour to examine the remaining protease activity.

The results are shown in the following table.

Table 6

Influence by metal ions

Metal ion	Remaining activity (%)
None	100
Ag^+	11
Cu^{2+}	44
Zn^{2+}	61
Co^{2+}	63

Fe^{2+}	76
Mn^{2+}	95
Ca^{2+}	100
Mg^{2+}	100

The present enzyme was extensively inhibited by Ag^{+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Fe^{2+}. Above all, when inhibited with Ag^{+}, only 10% of the protease activity was survived. The present enzyme was not inhibited by Mg^{2+} or Ca^{2+} at all.

Example 15

Substrate specificity

Casein (Hammarsten), dimethylcasein, gelatine, hemoglobin, bovine serum albumin, and ribonuclease were employed as the substrate proteins to measure the proteolytic activity by the modified Anson method. Azocasein and azoalbumin were used as the azoprotein modifying proteins.

The results are shown in the table below.

Table 7

Substrate specificity

Substrate (1%)	Hydrolysis rate (%)
Casein (Hammarsten)	100
Dimethylcasein	93
Gelatine	53
Hemoglobin	18
Bovine serum albumin	17
Ribonuclease	0
Azocasein	100
Azoalbumin	20

The enzyme of the present invention decomposes well

high molecular proteins and denaturated proteins such as casein and dimethylcasein, and also decomposed gelatine as the collagen denaturated protein in about 50% on the basis of casein. The enzyme scarcely acted on the other natural proteins, and it did not act particularly on ribonuclease.

Example 16

Enzyme reaction kinetics with casein

The Lineweaver-Burk plots at various temperatures from 5 to 40℃ were obtained with solutions containing 0.05 - 1% of casein as the substrate. The plots are shown in Figure 17, in which the upper graph (A) illustrates the change in 1/v value in the range of 0 to 2.0, and the lower graph (B) illustrates the change in 1/v value in the range of 0 to 0.2. In the figure, □ represents the plots at 5℃, ◇ at 10℃, ○ at 20℃, △ at 30℃, and ▽ at 40℃.

The kinetic constant of the enzyme reaction was obtained from the Lineweaver-Burk plots as shown in the table below.

Table 8

Substrate specificity

Substrate (1%)	Hydrolysis rate (%)
Casein (Hammarsten)	100
Dimethylcasein	93
Gelatine	53
Hemoglobin	18
Bovine serum albumin	17
Ribonuclease	0
Azocasein	100
Azoalbumin	20

As is apparent from the table, the enzyme exhibited a reaction rate of the Michaelis-Mentne type for the concentration of casein. The Km value decreased and the Vmax value increased with the increase of temperature. The

enzyme activity was inhibited in a high concentration of casein at 5℃ and 10℃. In general, the enzyme tends to be inhibited in an excessive concentration of a substrate. However, the enzyme did not inhibited in 1% of casein at an increased reaction temperature. This is considered due to the increase of the Kcat value by the approach to the optimal temperature and little inhibition by the decomposed product of casein. It is also considered from the Lineveaver-Burk plots that the inhibition mode by temperature is of a mixed type. That is, it is considered that the influence of temperature is a non-competitive or uncompetitive inhibition, and the influence of the decomposed product of casein is a competitive inhibition.

What is claimed is:

1. A psychrophilic protease having the following physicochemical properties:

- Specific activity and substrate specificity: it acts on casein and dimethylcasein to decompose them but does not act on ribonuclease;

- Optimal temperature: it has an optimal acting temperature at about 40℃; and

- Temperature stability: under the condition of storage at pH 7 for 1 hour, it is scarcely inactivated at a temperature up to 30℃, but at 40℃ it loses about 40% of the activity, and at 50℃ it is rapidly inactivated so that the activity is completely abolished in about 15 minutes.

2. A psychrophilic protease as claimed in claim 1, further having the following physicochemical properties:

- Optimal pH: it acts optimally at pH 7.5; and

- Stable pH range: it is stable at a pH in the range of 6.0 - 10.0 under the condition of storage at 20℃ for 1 hour.

3. A psychrophilic protease as claimed in claim 1, wherein the protease has a molecular weight of about 38 kDa as measured by SDS-PAGE and gel filtration methods.

4. A psychrophilic protease as claimed in claim 1, wherein the protease has an isoelectric point of about 4.5 as measured by isoelectric focusing.

5. An isolated microorganism belonging to Flavobacterium balustinum, which is capable of producing the protease as claimed in any one of claims 1 to 4.

6. An isolated microorganism belonging to Flavobacterium balustinum as claimed in claim 5, wherein

the microorganism preferably grows at a temperature in the range of 10 to 20 ℃.

7. An isolated microorganism belonging to Flavobacterium balustinum as claimed in claim 5, which is Flavobacterium balustinum P104 (FERM BP-5006).

8. A process for preparing a psychrophilic protease, comprising the steps of:

culturing Flavobacterium balustinum as claimed in claim 5, and

collecting the psychrophilic protease as claimed in claim 1 from the culture.

9. A process for preparing a psychrophilic protease, comprising the steps of:

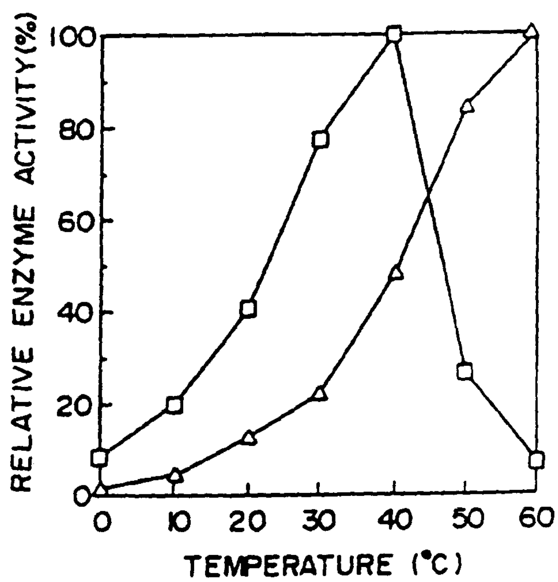
culturing Flavobacterium Balustinum as claimed in claim 6, and

collecting the psychrophilic protease as claimed in Claim 1 from the culture.

10. A process for preparing a psychrophilic protease, comprising the stpes of:

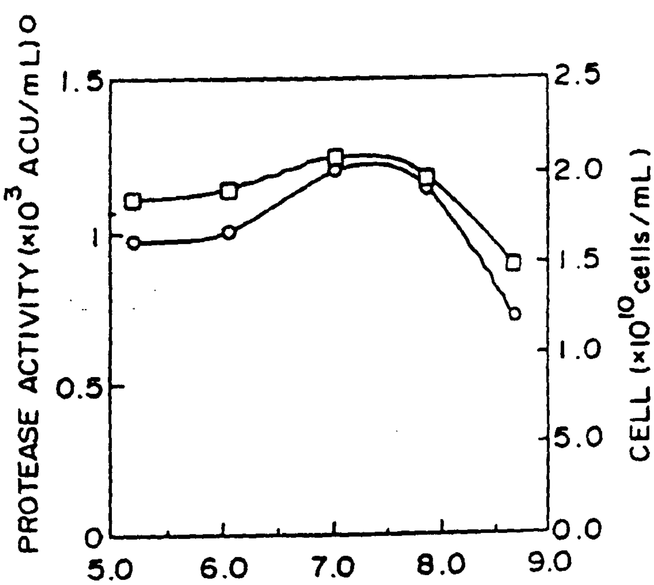
culturing Flavobacterium Balustinum as claimed in claim 7, and

collecting the psychrophilic protease as claimed in Claim 1 from the culture.



← P104 Protease

F I G. 1



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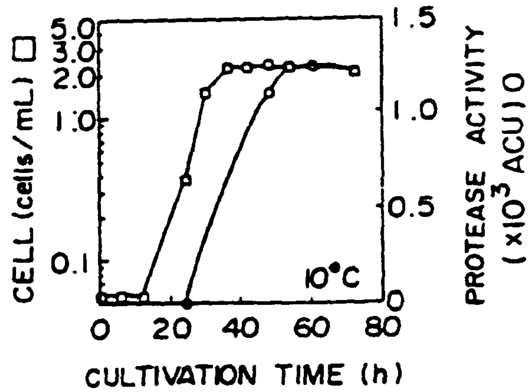


FIG. 3 (A)

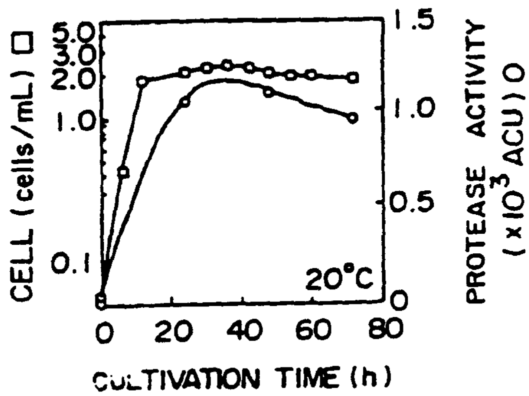


FIG. 3 (B)

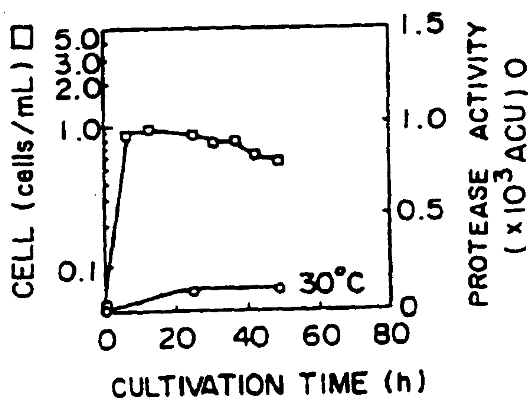
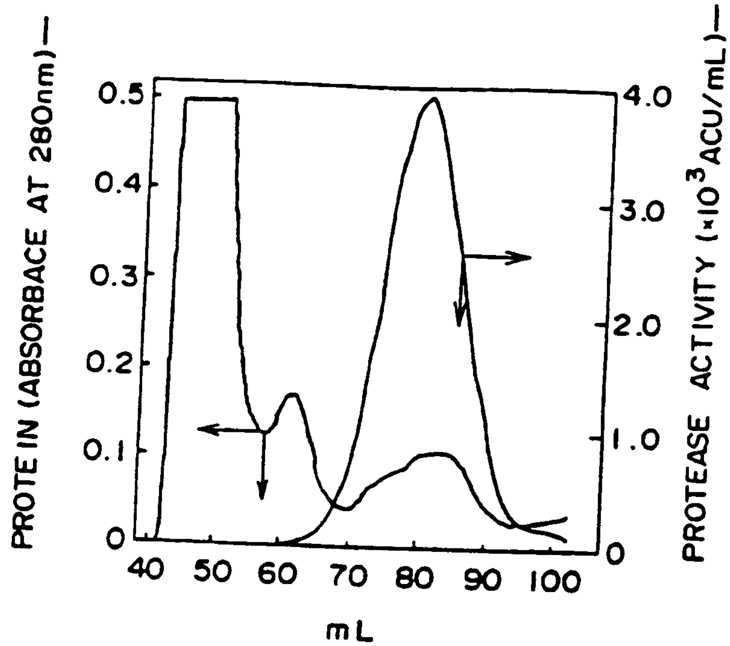


FIG. 3 (C)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0.3
0.2
0.1
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40 50 60 70 80 90 100
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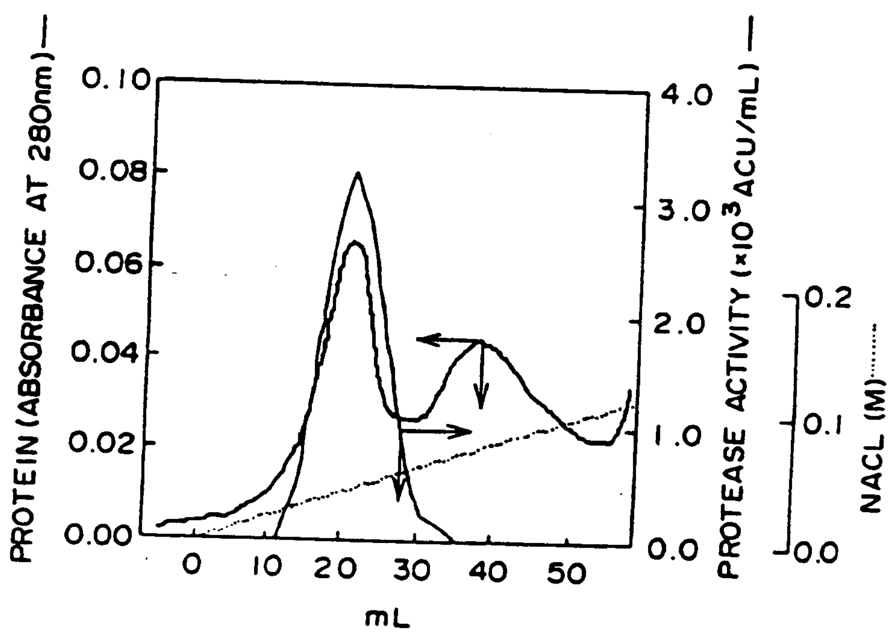
3.0
2.0
1.0
0

FIG. 4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3.0
2.0
1.0
0.0
0.2
0.1
0.0
NACL (M)

FIG. 5

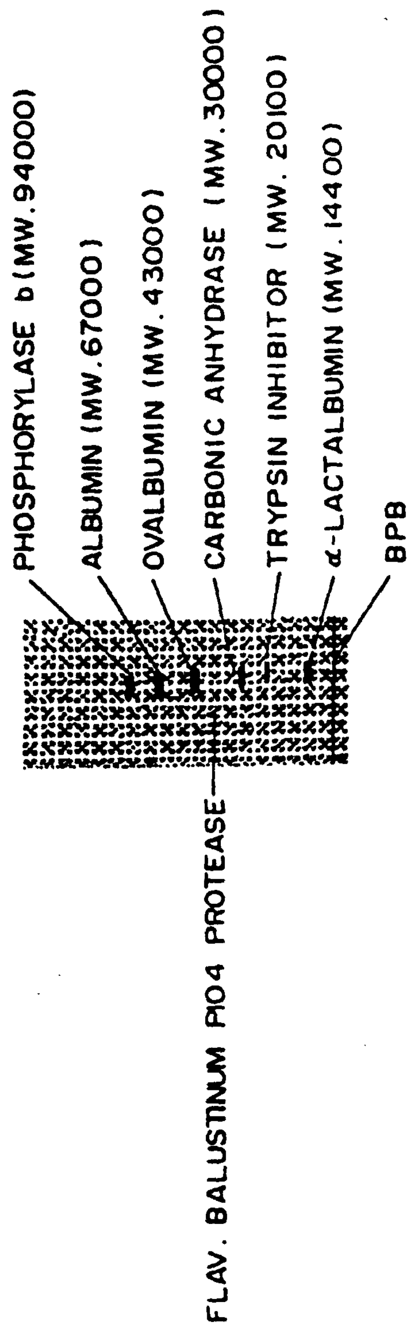
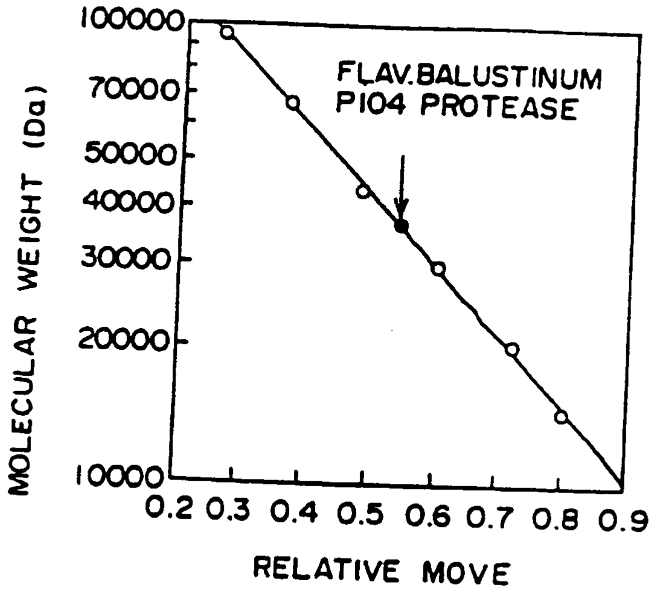
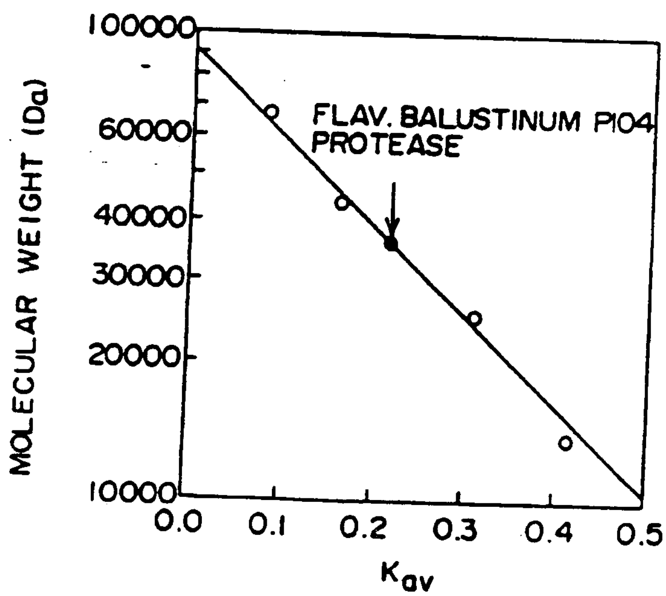

PHOSPHORYLASE b (MW. 94000)
ALBUMIN (MW. 67000)
OVALBUMIN (MW. 43000)
CARBONIC ANHYDRASE (MW. 30000)
TRYPSIN INHIBITOR (MW. 20100)
α-LACTALBUMIN (MW. 14400)
BPB
FLAV. BALUSTINUM P104 PROTEASE

FIG. 6



100000
70000
50000
40000
30000
20000
10000
MOLECULAR WEIGHT (Da)
FLAV.BALUSTINUM
P104 PROTEASE
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
RELATIVE MOVE

FIG. 7



100000
60000
40000
30000
20000
10000
MOLECULAR WEIGHT (Da)
FLAV. BALUSTINUM P104
PROTEASE
0.0 0.1 0.2 0.3 0.4 0.5
Kav

FIG. 8

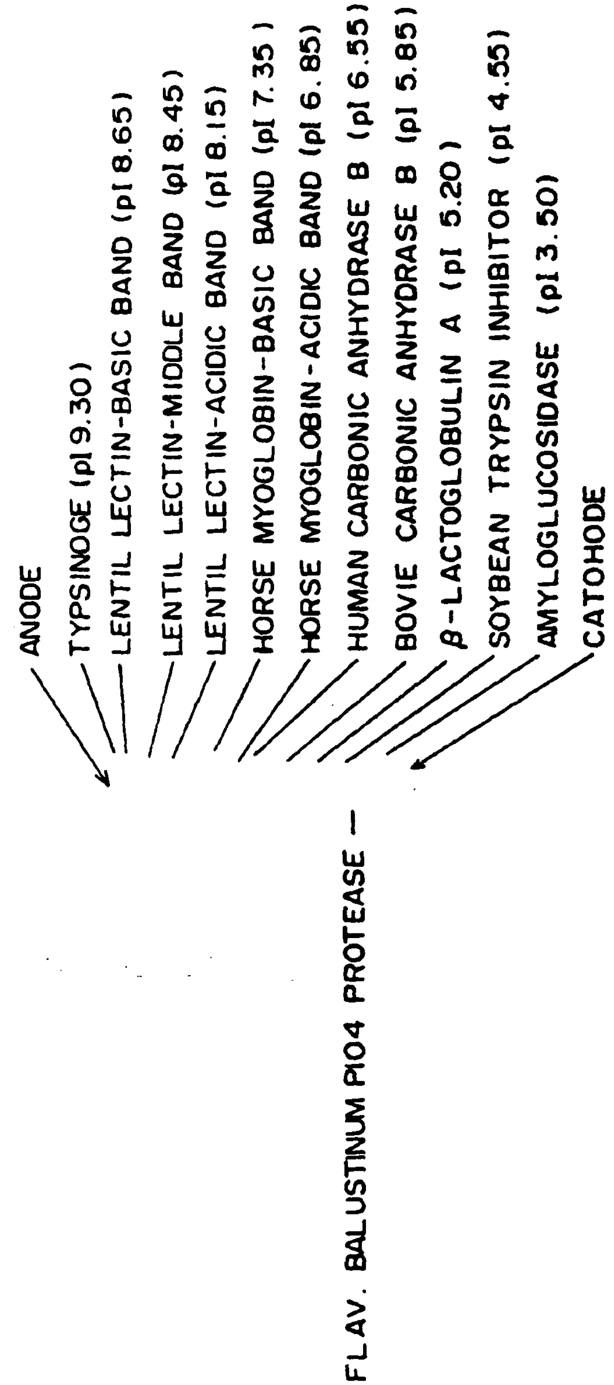

ANODE
TYPSINOGE (pI 9.30)
LENTIL LECTIN-BASIC BAND (pI 8.65)
LENTIL LECTIN-MIDDLE BAND (pI 8.45)
LENTIL LECTIN-ACIDIC BAND (pI 8.15)
HORSE MYOGLOBIN-BASIC BAND (pI 7.35)
HORSE MYOGLOBIN-ACIDIC BAND (pI 6.85)
HUMAN CARBONIC ANHYDRASE B (pI 6.55)
BOVIE CARBONIC ANHYDRASE B (pI 5.85)
β-LACTOGLOBULIN A (pI 5.20)
SOYBEAN TRYPSIN INHIBITOR (pI 4.55)
AMYLOGLUCOSIDASE (pI 3.50)
CATOHODE
FLAV. BALUSTINUM P104 PROTEASE

FIG. 9

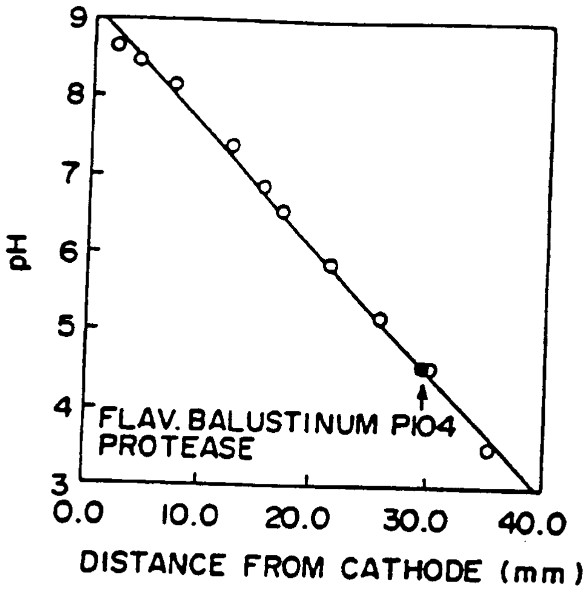

9
8
7
6
5
4
3
pH
FLAV. BALUSTINUM P104
PROTEASE
0.0
10.0
20.0
30.0
40.0
DISTANCE FROM CATHODE (mm)

FIG. 10

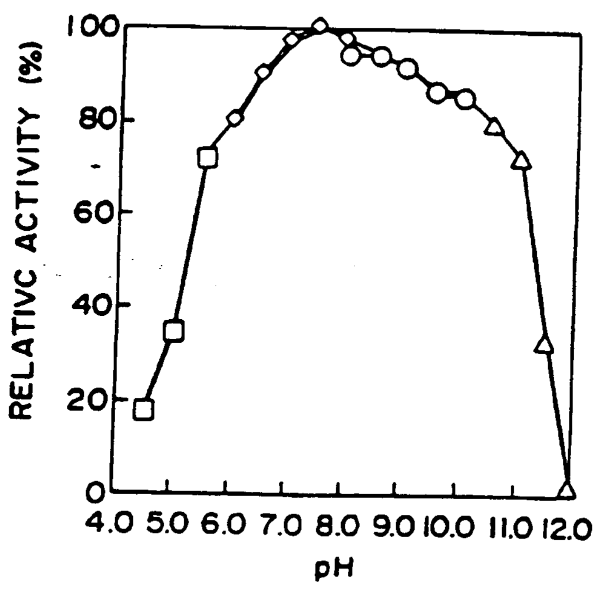

100
80
60
40
20
0
RELATIVC ACTIVITY (%)
4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0
pH

FIG. 11

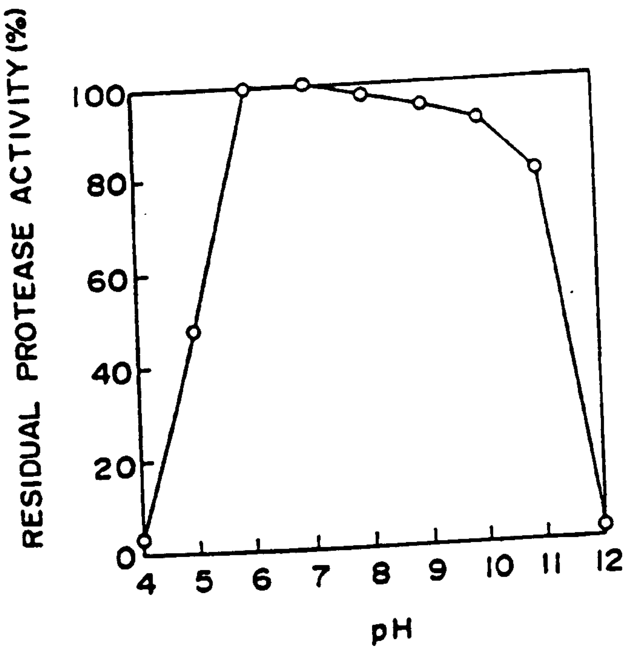

RESIDUAL PROTEASE ACTIVITY (%)
100
80
60
40
20
0
4 5 6 7 8 9 10 11 12
pH

FIG. 12

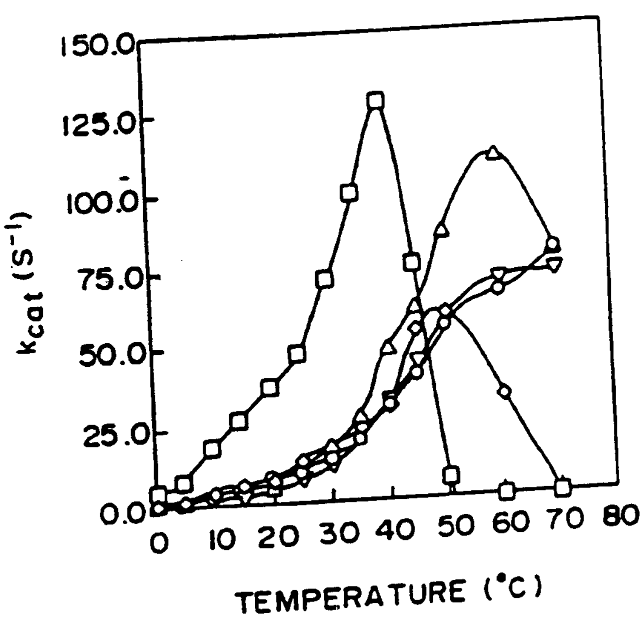
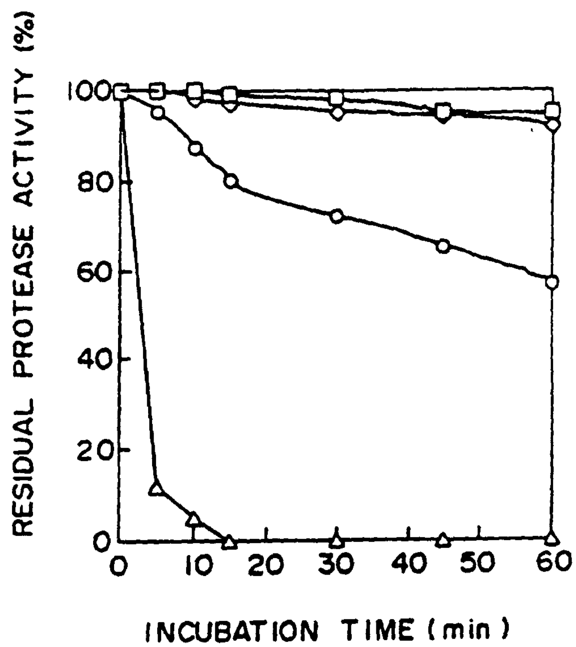
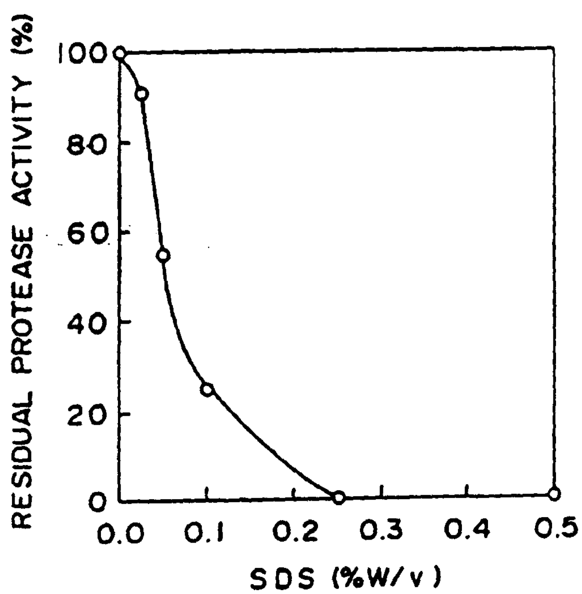

150.0
125.0
100.0
75.0
50.0
25.0
0.0
k_{cat} (S^{-1})
0 10 20 30 40 50 60 70 80
TEMPERATURE (°C)

FIG. 13


RESIDUAL PROTEASE ACTIVITY (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
INCUBATION TIME (min)

F I G. 14


RESIDUAL PROTEASE ACTIVITY (%)
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
SDS (%W/v)

F I G. 15

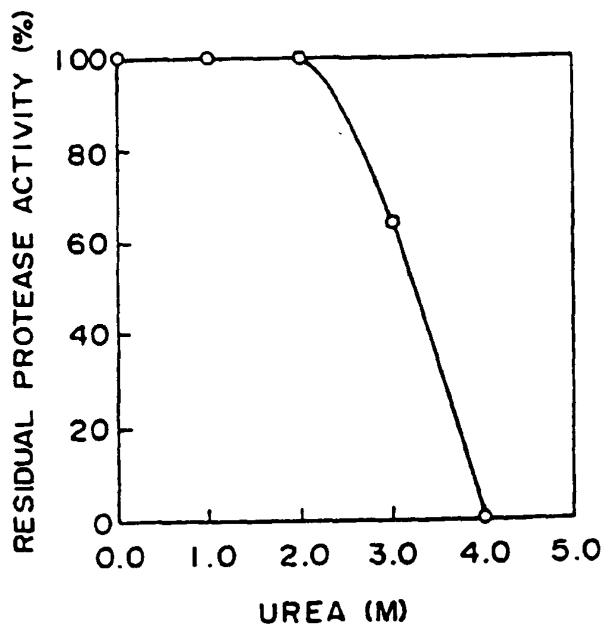
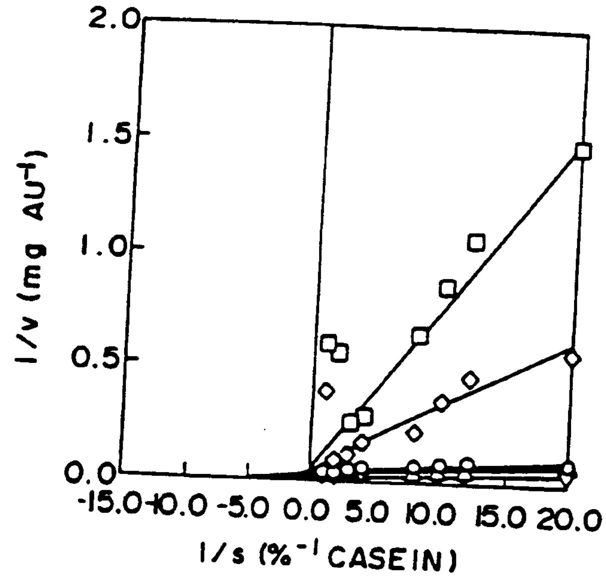
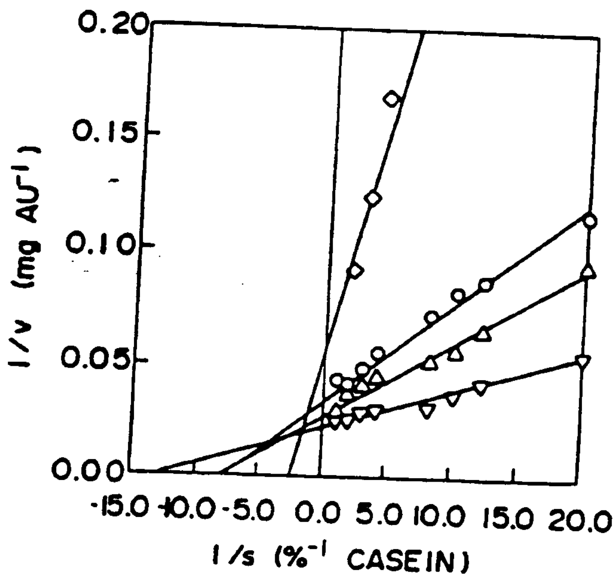

RESIDUAL PROTEASE ACTIVITY (%)
100
80
60
40
20
0
0.0
1.0
2.0
3.0
4.0
5.0
UREA (M)

FIG. 16



F I G. 17(A)



F I G. 17(B)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02115

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/48, 9/52, 1/20; C12P 21/04
US CL :435/252.1, 212, 220, 71.2, 850
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.1, 212, 220, 71.2, 850

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, DIALOG, MEDLINE, WPIDS.
Search terms: cold, psychrophil?, flavobacterium, balustinum, enzym?, protease?, proteinase?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JUAN et al. Produccion de Proteasa Extracellular por una Bacteria Criofila de Agua Dulce. Revista Asociacion Argentina de Microbiologia. 1976, Volume 8, Number 1, pages 8-13, especially page 8.	1-4
X	DOUSSET et al. Action des Proteases des Bacteries Psychrotrophes du Lait sur la Qualite des Produits Laitiers. Industries Alimentaires et Agricoles. May 1986, pages 325-333, especially page 329.	1-4
Y	MILLIERE et al. An Inventory of Peptide hydrolases and Arylamidases in *Flavobacterium II* b. Journal of Applied Bacteriology. 1985, Volume 59, pages 459-468, especially page 461.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 MAY 1996	24 MAY 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	IRENE MARX
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02115

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAGDOUB et al. Flavor Development acceleration in Ras Cheese Slurry using Bacterial Protease. Journal of Dairy Science. 1991, Volume 74, Suppl. 1, page 127, abstract D139, see entire abstract.	1-10
X	MARGESIN et al. A comparison of Extracellular Proteases from Three Psychrotrophic strains of *Pseudomonas Fluorescens*. Journal of General Applied Microbiology. 1992, Volume 38, pages 209-225, especially pages 211-212.	1-4
X	MARGESIN et al. Characterization of a metalloprotease from psychrophilic *Xanthomonas maltophila*. FEMS Microbiology Letters. 1991, Volume 79, pages 257-261, especially page 260.	1-4
X	JOOSTE et al. The Significance of Flavobacteria as Proteolytic Psychrotrophs in Milk. Milchwissenschaft. October 1986, Volume 41, Number 10, pages 618-621, see entire document.	1-10
X	MAGDOUB et al. Utilization of psychrotrophic bacterial protease for acceleration of flavor development in Ras Cheese Slurry. Cultured Dairy Products Journal. November 1991, Volume 26, Number 4, pages 24-28, especially page 25.	1-10
X	ISMAIL et al. Production of Proteases by Some Dairy Psychrotrophic Bacteria. Annals Agricultural Science. 1991, Volume 36, Number 2, pages 525-534, especially page 527.	1-10